

Progress on pollen-stigma compatibility in *Corylus* (hazelnuts): a review

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Abstract: The genus *Corylus* is an important member in the Betulaceae family of the order Fagales. Self-incompatibility is one of the choke points in breeding and hazelnut production. This review describes, the progress on pollen-stigma compatibility in *Corylus*, including basic reproductive characteristics, S-alleles testing and their applications, inter-specific hybridization, self-compatibility in wild species, and recent molecular research on the genetic mechanism of compatibility. Compared with other species, progress on pollen-stigma compatibility (sporophytic self-incompatibility, SSI) in *Corylus* is slow, especially those in the Brassicaceae family. De novo approaches should be used to target the unique reproductive characteristics of hazelnuts. The continuous improvement of technological platforms based on molecular biology should broaden the pathways in investigating compatibility in *Corylus*. In addition, research should be conducted on overcoming self- and cross-incompatibility, and approaches based on biological, chemical, and physical principles should be introduced. Both theoretical and practical study of compatibility in *Corylus* should be devoted to change the self-incompatibility trait into a benefit in the species evolution and nut quality establishment.

Key words: SSI; S-locus; basic mechanism; breeding; pollination

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Introduction

The genus *Corylus* is a member of the Betulaceae family of the order Fagales and includes several species. Hazelnuts have multiple uses and are sold on two main markets (the in-shell market and the kernel market). The in-shell market accounts of 5% to 10% of the world hazelnut crop. The remaining 90% to 95% are cracked and the kernels are sold to the processors such as bakers, candy makers, and oil manufacturers. Hazelnut production has been based primarily on selections from the wild over a very long history. Although a few cultivars have been selected from open-pollinated seedlings over the years, breeding programs to develop new and improved cultivars were not established until the 1960s in the United States, Italy, and France; the 1970s in Spain (Mehlenbacher 1990); and the 1980s in China (Liang et al. 1994). Today, 80% of the world hazelnut crop is still concentrated in Turkey and Italy; the remaining 20% comes from the United States, Azerbaijan, Spain, Iran, Georgia, and China (Fideghelli and De Salvador 2009). Management techniques in commercial orchards have been improved remarkably in recent years, but genetic improvements have not kept pace with the large economic value of this urgently needed crop. One of the choke points is the reproductive characteristics of *Corylus*. Hazelnut cultivars express sporophytic self-incompatibility and are also cross-incompatible in many combinations (Schuster 1924; Thompson 1979a). Incompatibility prevents making many desirable crosses and dictates their direction in many breeding programs (Mehlenbacher 1997a; Pomper et al. 1998). And also, in commercial orchards, one or more pollinators must be included to ensure good nut set on the main cultivar. So it is necessary to test the compatibility between the main and the pollinator cultivars in the orchards and to understand the basic mechanism of self-incompatibility in hazelnuts. New techniques, especially the molecular biology approaches, can be applied to accelerate the exploration and manipulation of this old but special characteristic in present and future cultivars.

Reproductive characteristics in *Corylus*

The hazelnut is monoecious, protandrous, and wind-pollinated (Lagerstedt 1975; Thompson 1979a). Self-incompatibility is a specific mechanism that prevents self-fertilization and encourages fertilization by genetically unrelated individuals (Nasrallah 1993). Two main forms of self-incompatibility exist, gametophytic (GSI) and sporophytic (SSI) (Matton et al. 1994). Incompatibility in cultivated hazelnuts (*Corylus avellana* L.) was first reported by Schuster (1924) and Johansson (1927). Hazelnuts express sporophytic self-incompatibility controlled by a single S-locus with multiple alleles (Thompson 1979a; 1979b). In female flowers, stylar S-alleles are codominant and pollen alleles are either codominant or dominant (Thompson 1979b; Mehlenbacher and Thompson 1988). Heslop-Harrison et al. (1986) reported that S-factors in *C. avellana* were held in the pollen wall, and it was possible that they formed one component of the poral proteins that were of sporophytic origin (from the tapetum).

Pollen in other sporophytically incompatible families is tricellular, such as Brassicaceae and Asteraceae, whereas *C. avellana* pollen is bicellular, and the stylar surface is covered with dry papillae, which is a characteristic of the sporophytic self-incompatibility (SSI) system (Heslop-Harrison et al. 1986; Hampson et al. 1993). In SSI, if the same S-allele expressed in a pollen grain was also expressed in the recipient pistil, pollen-tube growth was arrested on the stigmatic surface (Hampson et al. 1993; Matton et al. 1994). Scanning electron microscopy showed that reduced germination and coiled and bulbous pollen tubes were characteristics of incompatible reactions in hazelnut (Hampson et al. 1993; Thompson et al. 1996). Incompatible pollen may hydrate and germinate on the stylar surface as the compatible pollen does. However, no tubes were observed to penetrate into the style. Compatible pollen germinates well and the tubes penetrate the stigmatic surface producing a mass of long parallel tubes when viewed under a fluorescence microscopy (Mehlenbacher 1997a; Zhai et al. 2009).

S-alleles testing and their applications

Pollen-stigma compatibility relationships were first reported by Thompson (1979b) and then the method of view the germinated pollen tubes under the fluorescence microscope was introduced. A total of 11 S-alleles have been identified amongst 36 cultivars, of which one or both S-alleles have been established. Mehlenbacher and Thompson (1988) reported 11 additional alleles from *C. avellana* and the interspecies hybrid of Chinese Trazel Gelatly 4#. Dominance relationships of S-alleles have been determined in both pistil and pollen. In the pistil, all alleles exhibited independent action (co-dominance), whereas in the pollen, alleles exhibited either dominance or co-dominance. The dominance relationship was linear with 7 levels reported. Another four alleles were identified in 1997 and the dominance relationship was revised into a linear dominance hierarchy consisting of

eight levels as shown in Fig. 1 (Mehlenbacher 1997a).

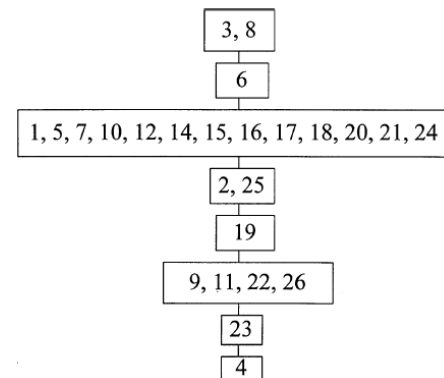


Fig. 1 Dominance hierarchy of S-alleles in hazelnut pollen (Mehlenbacher 1997a). Alleles are dominant to the alleles below them and codominant with those at the same level.

At the fourth international congress on hazelnuts in 1997, Mehlenbacher detailed the method on using the fluorescence microscopy to determine if the cross was compatible or incompatible (Mehlenbacher 1997b). The undetermined cultivars were tested with those having the known alleles. A single incompatible reaction and several compatible reactions indicated the presence of one known and one unknown allele or homozygosity. The compatibility of crosses can be determined in less than 24 hours using female inflorescences from bagged branches, frozen pollen, and fluorescence microscopy. In the several years that followed, similar efforts were carried out by researchers at Oregon State University and in many other countries. The method of fluorescence microscopy was widely used in S-alleles investigation (Erdoğan et al. 2005; Mehlenbacher and Smith 2006; Vicoli et al. 2009) and the pollinator selection for orchards (Okay and Ayfer 1994; Pedica et al. 1997; Beyhan and Odabas 1997; Grau et al. 2001; Ghanbaril et al. 2004). To date, 31 unique S-alleles have been identified in hazelnuts (Mehlenbacher 2009).

Inter-specific hybridization in *Corylus*

Liang et al. (1994) began their inter-specific cross on hazelnuts in 1980 in China. Their results showed that characteristics in the cross compatibility and the hybrid fertility differed greatly based on different parent combinations. The compatibility and fertility of the combination *C. heterophylla* × *C. avellana* were higher than other combinations, and the released inter-specific hybrids in China were the advanced seedlings of this combination (Zhang et al. 2005). To better understand the relationships among seven *Corylus* species and improve their possible use in breeding, Erdoğan and Mehlenbacher (1997) crossed them in all combinations. The results showed that the average percent cluster set varied among genotypes and the reciprocal differences were common. The European hazelnut *C. avellana* showed fairly good set with *C. colurna*, and the North American species *C. cornuta* and *C. californica* were highly cross-compatible, but

both showed lower degree of compatibility with *C. americana*. The Asian species *C. sieboldiana* and *C. heterophylla* were not compatible with each other. In other research, they reported the intercrossed results in eight hazelnut species that are shown in Fig. 2. The successful combination were *C. californica* × *C.*

avellana, *C. chinensis* × *C. avellana*, *C. americana* × *C. heterophylla*, *C. cornuta* × *C. heterophylla*, *C. californica* × *C. columbia*, and *C. americana* × *C. sieboldiana*, but the reciprocal crosses were not compatible. There were also many examples of unilateral incompatibility (Erdoğan and Mehlenbacher 2000).

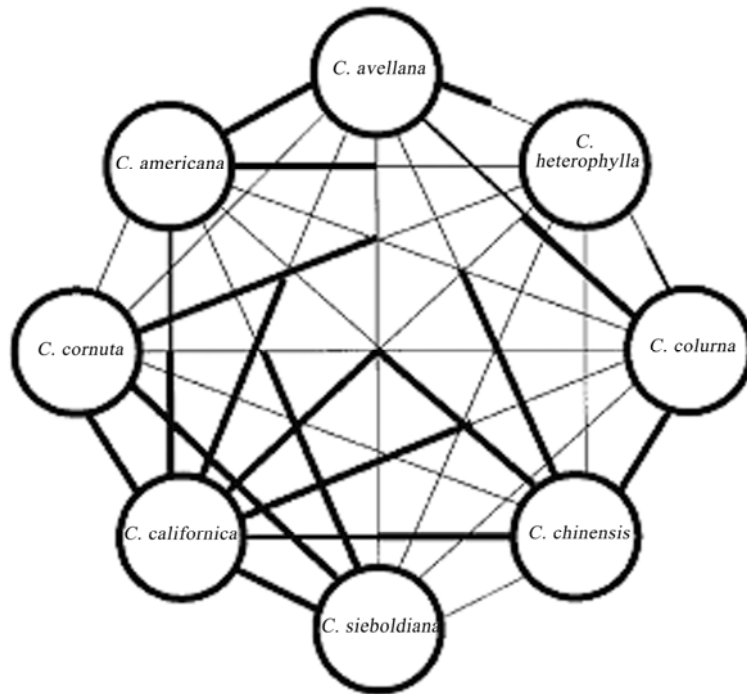


Fig. 2 Compatibility of inter-specific crosses in *Corylus* based on percent cluster set (Erdoğan and Mehlenbacher 2000). The width of the lines indicates the amount of set: thin 0% to 5%, medium 5.1% to 10%, and thick 10.1% to 25%. Single lines with two widths indicate reciprocal differences in the set of crosses.

In 2001, Erdoğan and Mehlenbacher reported their study of the incompatibility in wild *Corylus* species using fluorescence microscopy. The result indicated that sporophytic incompatibility existed in wild *Corylus* species and a large number of S-alleles were involved. Both compatible and incompatible reactions were observed in intra- and inter-specific pollinations with some reciprocal differences and clearly incompatible reactions observed in nearly all self-pollinations. In our group, five *Corylus* species, including *C. heterophylla* Fisch. (var. 9), *C. mandshurica* Maxim. et Rupr. (var. 1), *C. chinensis* Franch., *C. avellana* L. (cv. Ennis)

and *C. heterophylla* × *C. avellana* (cv. 82-11) were used to investigate the compatibility of the inter-specific hybridizations and self-pollinations among them (Zhai et al. 2009). The result showed that the inter-specific pollinations of *C. heterophylla* were all compatible and the same with the reciprocal crosses. *C. mandshurica* could be used as mater parent with all the other species but it was not compatible in some combinations when used as pollen parent. Unilateral inter-specific incompatibility presented in six combinations is shown in Table 1.

Table 1 Compatibility of inter-specific hybridization and self-cross in *Corylus* (Zhai et al. 2009)

Maternal parent	Pollen parent				
	<i>C. heterophylla</i> × <i>C. avellana</i> (82-11)	<i>C. avellana</i> (Ennis)	<i>C. heterophylla</i> (var. 9)	<i>C. mandshurica</i> (var. 1)	<i>C. chinensis</i>
<i>C. heterophylla</i> × <i>C. avellana</i> (82-11)	×	√	√	×	×
<i>C. avellana</i> (Ennis)	√	×	√	×	×
<i>C. heterophylla</i> (var. 9)	√	√	√	√	√
<i>C. mandshurica</i> (var. 1)	√	√	√	√	√
<i>C. chinensis</i>	×	√	√	×	×

Self-compatibility in wild *Corylus* species

Wild *Corylus* species play an important role in breeding work and germplasm research. It was necessary to introduce specific

traits from wild *Corylus* species for the improvement of the existing hazelnut cultivars (Thompson et al. 1996). Reports of self-compatibility were mainly discovered in wild *Corylus* species with several exceptions. Self-pollination of the *Corylus avellana* L. cultivars in 1988 and 1990 by Mehlenbacher and

Erdoğan (1991) revealed the existence of partial self-compatibility in ‘Tombul’ and ‘Montebello’. Percent cluster set in these cultivars averaged 44% and 20%, respectively, and the number were increased obviously when crossed with other pollens. In their 2001 study on wild species, Erdoğan and Mehlenbacher reported that under the fluorescence microscopy, one of four *C. californica* selections (Tombul) and two of three *C. columna* selections (X_{11} and X_{13}) were observed to be compatible on its own stigma. The controlled self-pollinations in the field of the three selections set well but showed high frequency of blanks. The results were consistent with their 1991 study. In studying the cutleaf hazelnut, Mehlenbacher and Smith (2006) found a new S-allele (S_{28}) that appeared to be a style-part mutation of S_{30} . Self-compatibility was observed in the genotypes when S_{28} was paired with a second allele at a lower position in the dominance hierarchy (Mehlenbacher and Smith 2006; Mehlenbacher 2009). Similarly, the results in our group showed that the wild species *C. heterophylla* and *C. mandshurica* were self-compatible, due to the adaptability of the wild environment (Zhai et al. 2009). Therefore, the self-compatibility of these species or selections deserves further investigation.

Molecular research on the genetic mechanism of compatibility in *Corylus*

As mentioned above, hazelnuts express the sporophytic type of self-incompatibility. SSI has been reported in Asteraceae, Betulaceae, Brassicaceae, Caryophyllaceae, Convolvulaceae, Sterculiaceae, and so on, but the molecular mechanisms were still partly unknown except for the Brassicaceae (Hiscock and Tabah 2003). Hampson et al. (1996) first reported the molecular mechanisms of SSI on hazelnuts and raised the question of whether the genome of *Corylus avellana* contains sequences homologous to the self-incompatibility gene of *Brassica*. With the hypothesis that the hazelnut genome contained homologous sequences with *Brassica*, the S-locus glycoprotein gene (*SLG*) of *B. oleracea* was used to probe blots of genomic DNA from hazelnuts. Unfortunately, weak hybridization of identical molecular weight with the *SLG* probe was detected and no hybridization was found with PCR-generated probes corresponding to the two conserved regions of the *SLG* gene. Based on these results, Hampson (1996) concluded that the SSI genes of *Brassica* and *Corylus* had either diverged greatly during evolution or are of independent origins. This conclusion was either correct or does not need to be proven by a series of correlative researches. Molecular research on the genetic mechanism of compatibility in *Corylus* has to be developed using *de novo* approaches in the long run.

The routine method of testing cross-compatibility in *Corylus* was to use fluorescence microscopy after crosses in the period of florescence (Mehlenbacher 1997b). The advantage of molecular biology that it is not restricted by the seasons and the age of the seedlings and so could be used in marker-assisted selection of young hazelnut seedlings years before the S-alleles of individual trees could be typed at flowering in the field. Molecular markers enabled selection strategies for rare allelic combinations desir-

able in future cultivars or for the development of universal pollinizers. RAPD and BSA methodologies were utilized to identify RAPD and SCAR markers for hazelnut S-alleles by Pomper et al. (1998), Bassil and Azarenko (2001), respectively. Many horticultural crops are highly heterozygous, the same with hazelnuts.

Therefore, in the two studies above, the F_1 progenies that was considered to represent a segregating population due to the high heterozygosity of the parents, could be bulked according to S-allele type and used in a RAPD screening. Both of the studies were based on Mehlenbacher’s result of the S-alleles and their dominance hierarchy in the bulked segregation. Pomper et al. (1998) identified two RAPD markers each for S_2 (OPI07₇₅₀) and S_1 (OPI14₁₇₀₀), OPI07₇₅₀ served as an excellent marker for the S_2 allele and was linked closely to this allele. Bassil and Azarenko (2001) identified one RAPD marker (OPN20₁₃₀₀) was linked to an S-allele and confirmed that OPI07₇₅₀ identified in Pomper’s study was an excellent marker for the S_2 allele. Two pairs of SCAR primers of OPI07₇₅₀ were made and amplified in the tested individuals. The sequence showed 28% identity at the amino acid level to a putative reverse transcriptase from *Oryza sativa* and to a reverse transcriptase domain from *Arabidopsis thaliana*.

As for other molecular markers, when Kuzmanović et al. (2009) studied the allele segregation at SSR loci for hazelnut accessions from landraces grown in the Latium region, in the combination of TGR \times NOC, only one of the SSR paternal alleles (A_7) was transmitted to the progeny at the locus CaT-B107. While the results of the reciprocal cross NOC \times TGR showed a normal segregation ratio of alleles A_4 and A_7 . They considered that the A_4 allele might be linked to an allele, other than S-locus, involved in the pollination control mechanism and expressed only in the pollen which caused the unilateral gametophytic incompatibility.

The Differential Display technique was applied by Marinoni et al. (2009) to investigate the gene expression in two developmental stages of styles/stigmas. The differentially expressed bands were sequenced and aligned in the database. Partial sequences showed homology degree with the transmembrane serine-threonine kinase receptor of *Brassica oleracea*. Therefore, primers were designed on conserved regions of serine-threonine kinase receptors and four differentially expressed fragments were isolated from stigmas at full bloom. The results showed that one fragment was homologous to a kinase receptor, three were homologous to kinase proteins. Their study may not give Hampson’s question an absolutely “Yes” answer, but at least, found a new way out to investigate the female determinant of self-incompatibility.

Prospect

More effort has been devoted into the study of the compatibility in *Corylus*, while the progress on this species is still at the threshold comparing with other species, especially those in Brassicaceae. Although *Corylus* was considered to be a model species for the Betulaceae with a small haploid genome and a relatively

short life cycle (less than five years to first flowering) (Mehlenbacher 2009), the woody plant still had a longer life span compared with *Brassica*. Furthermore, the floral biology of hazelnuts is very particular as mentioned above. Female flower clusters can not be distinguished from leaf buds until the red stigmatic styles protrude out at anthesis and the tiny styles are joined at their base by a minute ovarian meristem.

Given that the procedures of many experiments and treatments used in *Brassica* could not be carried out, alternative methods should be used to target the unique traits of hazelnuts. In *Corylus*, the construction of crossing and back-crossing populations goes on for several years, while BSA could be used in the present populations with known S-locus information. The molecular markers and the converted SCAR markers linked to S-locus could be used as genetic markers in identifying the allele expressing the compatibility in hazelnuts and helping marker-assisted selection of desired S-genotypes.

Corylus is a non-sequenced species with little homologous sequences for reference, but differential display technique (Liang and Pardee 1992), the following high-throughput technologies such as suppressive subtraction hybridization (SSH) (Diatchenko et al. 1996), serial analysis of gene expression (SAGE) (Velculescu et al. 1995; 2000) and digital gene expression (van't Veer et al. 2002; Feng et al. 2010) would be carried out to develop spatially and temporally specific gene-expression profiles in different development stages of the male and female determinant. Moreover, the continuous improvement of technological platforms based on the techniques above as well as proteomics, metabonomics, bioinformatics and system biology would broaden the pathways in clarifying pollen-stigma recognition reaction of self-incompatibility. Such studies should be powerful to provide a global view on the cellular responses for hazelnut to the particular reproduction traits.

In addition, some investigations have been conducted to overcome self-incompatibility in many other species but as yet hazelnuts have not been probed from this angle. The approaches based on biological, chemical and physical principles have been introduced in different taxa (Meng 1995) and studied comparatively (van Creijl et al. 1999; Chen et al. 2007). Treatments of stigma by heat, electricity, CO₂ and laser showed efficaciously in overcoming self- and cross incompatibility (Roggen et al. 1972; Roggen and van Dijk 1976; Dhaliwal et al. 1981; Li et al. 1995). Chemical controls also play very important roles in breaking down the incompatibility reaction on the pistil surface. Reports were focused on cytokinin, IAA, lectin, saccharide, quercetin and other chemical substances that were sprayed on the stigma before or right after pollination (Matsubara 1973; Sood et al. 1982; Sharma et al. 1983; Li et al. 2005). As for the pollen, the physical treatment mentioned above also feasible with modified conditions. Pollen treated with cycloheximid, saccharide and other substances could germinate on and penetrated into self-stigmas (Ferrari and Wallace 1977; Sharma et al. 1984). The mixed pollen or recognition pollen, by using killed compatible pollen along with live incompatible pollen, which could be one of the simplest means of overcoming barriers to fertilization (Sastri and Shivanna 1976; Pandey 1979).

For hazelnut production to thrive in the years ahead, one or more pollinators must be used in orchards and if necessary, mixed pollen with manual pollination should be applied to ensure good nut set. The cross practice between species and cultivars at the lab and in the field should be performed continuously. In addition, growth regulators and liquid fertilizers should also be tried out to improve the yield at florescence and in the growth period. In new cultivars breeding and selection, the first and foremost thing is to understand the basic mechanism of compatibility in *Corylus*. Methods based on biological, chemical, physical, and molecular theories should be tried out to overcome incompatibility between the desirable crosses. To sum up, the eventual objective of future compatibility study is to improve the present cultivars and ensure nut set for commercial use. It is very believable that with the rapid development of hazelnut varieties all around the world, the compatible trait serves not only as a handicap but also as a benefit in the species evolution and nut quality establishment.

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